Mycorrhizae of Ponderosa Pine in Nebraska Grassland Soils

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by R. W. Goss[1]

INTRODUCTION

Mycorrhizae as morphological structures consisting of root tissue and fungus mycelium have been known for many years and their significance has been the subject of much controversy. The early work on mycorrhizae of trees was largely based on observations, speculation and empirical tests. A thorough search of the literature, however, reveals convincing evidence that under certain conditions trees are benefited by the association of root and fungus which results in the structures termed mycorrhizae. In recent years this conclusion has been supported by the results of experimental studies under controlled conditions.

While the genus *Pinus* has been the subject of extensive mycorrhizal studies there are only a few scattered references to western yellow pine, *P. ponderosa,* and no detailed descriptive literature on the mycorrhizae of this species. *P. ponderosa* is indigenous to Nebraska and the one species most extensively grown in local nurseries for windbreaks and farm plantings. Similarly, there is little information on mycorrhizae in the Great Plains between the forested regions of the Mississippi River states and the Rocky Mountains. Because this predominantly grassland area is sparse in natural tree growth the individual tree as an ornamental or in groups as windbreaks becomes of great importance. Under the diverse soil conditions, and with relatively low rainfall and high evaporation and transpiration rates, the survival of transplants is relatively low. The possible role of mycorrhizae in influencing the survival rate has never been investigated in Nebraska.

The present study was undertaken to determine in a preliminary way the occurrence and significance of mycorrhizae on the production and survival of ponderosa pine in virgin grassland soils.

THE NATURE OF PINE MYCORRHIZAE

The literature on pine mycorrhizae is voluminous, scattered and often speculative and conflicting and no attempt will be made to present a critical review. Work in recent years, based upon more exact experimentation, has greatly enlarged our understanding of the mechanisms involved in the physiology of these host and fungus associations.

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Figure 1. Root-ball of 14-month-old ponderosa pine seedling growing in duff-soil mixture showing development of coralloid ectotrophic mycorrhizae with numerous rhizomorphs.

Nevertheless, every experiment opens up new questions and there is still much to learn about the relationship of mycorrhizal fungi to tree development. The following introductory statement is simply a brief summation of the outstanding characteristics of mycorrhizae as described in the literature.

The gross characteristics noticeable upon careful examination of the roots of pine trees have been known and recorded for many years. The relatively shallow, absorbing short roots of the pine in their native habitat are dichotomously branched and large numbers of these may be formed in coral-like groups termed coralloids. These thick, short roots are covered with a mantle of fungus mycelium, often white but which may be of various light colors turning brown with age. Sometimes the many branches are so campacted that they have a nodular appearance. Attached to the fungus mantle are often rope-like strands of hyphae, the rhizomorphs, which penetrate out through the soil (Figs. 1 and 2).

The absorbing short roots forming mycorrhizae are described as

Figure 2. Coralloid mycorrhizae on ponderosa pine seedling grown on inoculated Chestnut soil in greenhouse.

annual and ephemeral and are most abundant in the spring and fall during the periods of root growth. With rare exceptions the mycorrhizae are devoid of root hairs as contrasted with simple, unbranched non-mycorrhizal short roots which are abundantly supplied with root hairs. It should be remembered that the above characteristics, particularly with reference to the type and extent of dichotomous branching both with and without the fungus associate, will probably vary greatly with the species of pine being studied, with the species of fungus present and with the environmental conditions.

Fungi associated with pine mycorrhizae are those that normally inhabit the forest floor. Early workers observed and carefully traced the rhizomorphs connecting the mushrooms of the forest floor with the tree roots. These painstaking observations, however, did not prove a causal relationship. It was not until methods were devised of isolating the fungus directly from the mycorrhizae or from the rhizomorphs, growing it in pure culture and introducing it into cultures of aseptically growing pine seedlings with the resultant formation of mycorrhizae, that the causal relationship was proved.

Using this technique, it has been shown that many species of Hymenomycetes (mushrooms) are capable of forming mycorrhizae in association with pine roots. Other types of fungi have also been proven capable of forming mycorrhizae, but judging from the number of

successful tests the Hymenomycetes seem to be predominant. Such studies have provided important information about the fungi associated with mycorrhizae and synthetic cultures have proven to be a valuable technique in studying the nature of the physiological relationships.

The mycorrhizae of pine, and also of spruce, oak, elm, beech, hickory, chestnut, birch and others, are known as ectotrophic. Endotrophic types, where the fungus does not form a mantle and grows within the cells rather than intercellularly, are found on such trees as yellow poplar, maple and sweet gum.

In the ectotrophic type on pine the structure of the mycorrhizae is revealed, by microscopic examination of sections, to have certain definite characteristics. The fungus mantle may vary from a thin sheath, not visible under a dissection microscope, to a heavy mantle sometimes thicker than the cortex of the root. It is composed of either a loosely interwoven mass of hyphae or densely compacted strands of mycelium. The mantle is connected with a network of mycelium which penetrates through the intercellular spaces of the outer cortex, forming what is known as a "Hartig net" which is often so extensive as virtually to isolate the individual cells. The mycelium does not penetrate into the stele nor into the meristematic tissues of the root-tip although the mantle may cover the tip. There is hypertrophy of the cortical tissue which results in the root being thicker because of the increase in radial diameter and a decrease in longitudinal diameter of the individual cortical cells. There is usually a deposit described as a tannin layer just below the collapsed outer cortical cells and mantle.

The formation of mycorrhizae depends upon contact between the tree root and a fungus capable of penetrating the root and establishing an association. This may occcur by mycelial strands or rhizomorphs penetrating through the soil from established mycorrhizae on other roots and on roots of neighboring trees. Robertson (1954) reported that living seedlings with mycorrhizal infection were a good source of inoculum but that the detached roots of similar plants were not. However, there are many reported experiments where the inoculation of non-mycorrhizal seedlings with pine duff has resulted in beneficial effects associated with the resultant formation of mycorhizae. Successful development of mycorrhizae also has been demonstrated by the common nursery practice of transplanting mycorrhizae-bearing seedlings into new nursery soil a year or two before sowing seed. In neither case are mycorrhizal seedlings in direct contact with the new seedlings.

Robertson (1954) also presents evidence that mycorrhizal inoculum can be air borne. His seedlings from sterilized seed grown in pots of sterilized soil placed in the forest became mycorrhizal. He considered that in the case of *Boletus granulatus* the inoculum probably consists of basidiospores and refers to the common occurrence of contamination

in greenhouse experiments. He also suggests the possibility of chlamydospores or small sclerotia serving as aerial inoculum.

Infection of the emerging short roots has been considered to be caused by invasion by fungus mycelium from the soil and to be annual in nature. Long roots have been considered free of infection (Hatch and Doak, 1933). Robertson (1954) however, has described the development of intercellular mycelium in the long root. The extension of this cortical infection into the new long root in the spring results in the short roots becoming infected after they have broken out of the cortex, either directly by way of the mycelium in the cortex of the long root or by means of an external weft of mycelium. This concept of a perennial internal infection raises many questions regarding the interpretation of previous experimental work.

A modification of typical ectotrophic mycorrhizae has been reported, infrequently in America, in which the mycelium also occurs within the cells of the cortex and has been called ectendotrophic. As originally described this intracellular mycelium was considered to be digested by the cell prior to the formation of the intercellular net. Later papers have described a wide variation in the manner and incidence of this intracellular infection. It is difficult from the literature to define exactly, the line between a normal ectendotrophic mycorrhizae and intracellular infection by an ectotrophic fungus due to an upset in the physiological balance between root and fungus, with the fungus becoming the dominant partner.

In England, Miss Levisohn (1954) described an aberrant root development characterized by an haustorial type of infection within the cells of the cortex and this intracellular mycelium was not found to be digested. There was a very coarse intercellular net and complete absence of a fungus mantle. Brownish coarse strands of mycelium were found on the root surface. This type of infection resulted in forking of the short roots with the branches being of irregular length and produced on long, heavily infected, uneven "stalks." The mycelium which was isolated and found to produce similar infections in inoculation tests resembled the mycelium of *Rhizoctonia sylvestris*. Infections of the above type were harmful but the symptoms were variable with different species and under different soil conditions.

Pseudomycorrhizae is the term usually used to describe mycorrhizae with aberrant structural features associated with an adverse effect on the growth of the tree. Descriptions of pseudomycorrhizae have varied somewhat by different authors (Rayner and Neilson-Jones 1944; Hatch and Doak, 1933) but there is rather general agreement that they may be infections of either simple monopodially branched or dichotomously branched roots. They are dark as contrasted with the usual light colored mycorrhizae. The infection is intracellular and is not restricted to the cortical tissue, and the mycelium may form a pseudoparenchymatous mass within the cells. There are no root hairs,

no hypertrophy of the cortical cells, a complete absence of an intercellular net and no evidence of digestion of the hyphae. The fungus mantle is either absent or much thinner and does not occur over the tip of the root. Such short roots have been described as ceasing terminal growth earlier and some workers have noted the disappearance of the tannin layer which is conspicuous below the mantle of true mycorrhizae.

Robertson (1954) studied the distribution of pseudomycorrhizae and suggests that the short roots near the tip or the base of long roots are most frequently affected. He considers that true mycorrhizae may become infected and turn black. Latham, Doak and Wright (1939) considered that under field conditions most non-mycorrhizal short roots of pine are predominantly pseudomycorrhizal. Doak and Fisher (1935) report that pseudomycorrhizal as well as mycorrhizal short roots develop on *Pinus strobus, P. taeda, P. echinata* and *P. resinosa* during the first year's growth in nurseries in eastern United States. Doak (1934) reported the isolation of a fungus resembling *Rhizoctonia sylvestris* from roots of *P. taeda* with the occurrence of a mantle and Hartig net and also infection of the cortex of mother roots. No harmful effects were noted after 18 months and thus it could hardly be termed pseudomycorrhizal as it is generally agreed that pseudomycorrhizae have an adverse effect on growth.

The physiological phenomena involved in the formation of mycorrhizae is of the greatest importance to a full understanding of the whole mycorrhiza problem (Melin 1953). Unfortunately, space does not permit a comprehensive review of the many recent contributions to our knowledge of the subject. The real mechanism of the symbiotic action involved is not fully understood, but from the published experimental evidence some general conclusions and assumptions can be made (Slankis, 1958).

Ectotrophic and ectendotrophic mycorrhizae have been shown to be beneficial to both tree and fungus. This symbiosis is widespread and habitual among forest trees and when it does not occur both tree and fungus may suffer from nutritional difficulties under certain conditions. Mycorrhizae have been shown, by the use of radioactive isotopes, to increase the uptake of both organic nitrogen compounds and inorganic nutrients.

Evidence indicates the importance of exudates both by the roots and by the fungus mycelium in the development of mycorrhizae. The effect of roots on the microflora of the rhizosphere is well known. Experimental evidence attributes this effect to the root exudations. Many mycorrhizal fungi are deficient in certain growth promoting substances such as thiamine which may be provided by tree roots which have been shown to exude various organic substances and growth stimulating metabolites. Mycorrhizal fungi have in turn been shown to exude auxins which stimulate the production of simple or coralloid branched

root structures with hypertrophy of the cortical cells and inhibition of root hair development similar to true mycorrhizae. The same type of structures have been produced by several synthetic auxins as the roots of trees are very sensitive to auxin concentration with elongation being inhibited by high concentration and stimulated by low concentration. Studies with excised roots, however, do not provide the needed information on the transport and distribution of auxin in the root system.

Other factors in the soil environment have either direct or indirect effects upon the development of mycorrhizae. While mycorrhizae of trees are usually most abundant in acid soils, the optimum pH probably varies for different species of mycorrhizal fungi and has not been determined for some of them. Similarly, the occurrence of mycorrhizae has been associated with the lower temperatures of fall and spring, with good soil aeration and abundant moisture, but the exact requirements for many of the fungi involved have not been determined.

The formation of mycorrhizae has been shown to vary inversely with soil fertility, especially when related to the available nitrogen, phosphorus and potassium. The presence of certain vitamins and amino acids, for which some fungi are heterotrophic, influences their development and the effect of inhibitory substances produced by other microorganisms in the soil requires further study. Light exerts an indirect effect through its influence on the metabolism of the tree and the translocation of carbohydrates to the root which in turn affects the development of mycorrhizae. Recent studies have shown that the application of some biocides for the control of parasitic fungi or nematodes in nursery soil may destroy the beneficial mycorrhizal fungi (Hasckaylo and Palmer, 1957-(b), Wright 1957).

Much has been learned by the use of pure cultures and the study of excised roots, but a great deal more needs to be known regarding the physiological balance of tree root and fungus symbiont under natural conditions in the presence of the complex environmental factors living and non-living which exist in the soil.

TYPES OF MYCORRHIZAE IN NEBRASKA

Mycorrhizae most commonly encountered in Nebraska field observations and greenhouse experiments were of both the ecto- and ectendotrophic forms. They were found to have the same distribution as to soil, location and pine species.

Ectotrophic Mycorrhizae

The ectotrophic types were by far the most common in all specimens examined. They occurred on pine roots growing in the wide variety of soils used for shelterbelt plantings, in the sandy soils of the Nebraska National Forest at Halsey and in several nursery soils. By inoculation with a pine duff-soil mixture they became established, at

least for the duration of these experiments, in virgin grassland soils obtained from eastern Nebraska prairie, from Sandhill range land and from Chestnut soil of the western Nebraska tableland. Microscopic examination of these ectotrophic mycorrhizae revealed considerable variation in structure and type of mycelium, but for descriptive convenience they can be roughly divided into two types.

Type 1.—These mycorrhizae usually occurred as coralloids with a relatively loose white mantle and many attached rhizomorphs. The mantle sometimes covered the simple, unbranched short roots but more often occurred on the dichotomous branches where it sometimes appeared as a tent-like structure extending only a short distance down the "stalk" below the branches. Occasionally, single or multiple forked roots with only a sparse, inconspicuous mantle without rhizomorphs were present. In pots in the greenhouse the root-ball was often well covered with a dense, compact, discrete white fungus growth associated with the coralloid mycorrhizae and with many rhizomorphs (Figs. 1 and 2).

In stained sections (Fig. 3) the fungus mantle was found to vary from 15 to 50 microns in thickness. It was composed of relatively

Figure 3. Longitudinal section of Type 1 ectotrophic mycorrhiza with loose mantle of fine mycelium and intercellular net.

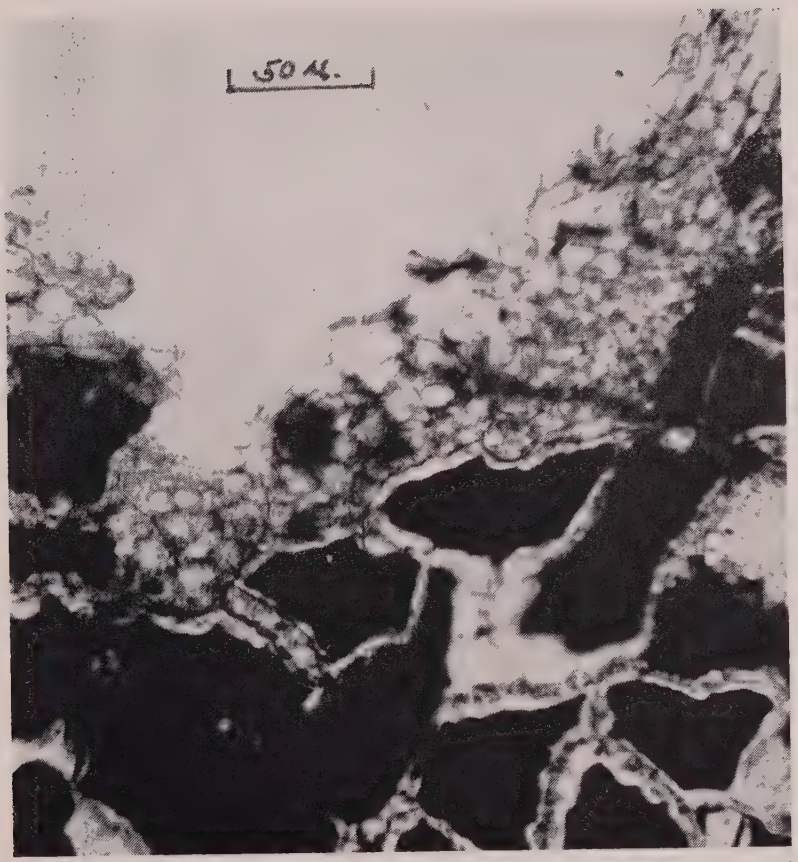

Figure 4. Longitudinal section of Type 2 ectotrophic mycorrhiza with mycelium of large bulbous cells in Hartig net and mantle.

loose interwoven strands of fine, septate, hyaline mycelium, 2.5-3.0 microns in diameter. There were often individual or multiple strands of hyphae projecting beyond the surface of the mantle. Clamp connections were clearly discernable in many of these hyphal strands. In many sections the hyphae of the mantle tended to break up into individual cells and the mantle was not always continuous. The mantle was usually present but was thinner over the tips of the short roots and was thickest in the crotch of the forked roots.

The mycelium of the mantle was connected with an extensive intercellular net usually two or three cells deep but sometimes extending throughout the cortex as far as the endodermis. It was never found in the endodermis nor in the meristematic tissues at the growing points. A tannin layer was usually present just beneath the mantle.

The cortex was hypertrophied with the individual cells being as wide as they were long, resulting in the thickening of the affected short root. Root hairs were rarely present and when they occurred arose from the epidermal cells and were only found on the "stalk" below the branches of the forked roots.

Type 2.—These ectotrophic mycorrhizae differed from Type 1 in that coralloids were seldom formed, there was no fungus mantle visible under the dissecting microscope and there were no rhizomorphs. Clamp connections were not observed.

In sections there was evident a thin, relatively loose mantle composed of irregularly septate, coarse mycelium with polyhedral cells 6 to 12 microns in size. The mantle was very thin or absent over the tips of the short roots. The intercellular net was composed of large, bulbous type cells (Fig. 4). There was a tannin layer and hypertrophy of the cortex. As with Type 1 there was no intracellular invasion. Type 2 was not as commonly encountered as Type 1 but was found in the same variety of soils and on Austrian, pinyon and white pine as well as on ponderosa.

Other ectotrophic types.—In some specimens there were variations of the above types but they occurred so rarely that they hardly warrant separate descriptions. For example, one specimen differed from the description of Type 1 only in the formation of the mantle. The mycelium of about 2.5 microns diameter appeared indistinguishable from Type 1 but formed a much looser mantle with the hyphae often at right angles to the surface and projecting beyond the mantle.

One specimen worthy of separate mention was from a 3-year-old Austrian pine growing in a commercial nursery and originating in a nursery in Wisconsin. The mantle of this specimen was covered with setae that were 50 to 70 microns long, about 2.5 microns in diameter at the base and 1.5 microns at the tip, with a cross wall near the base. The origin of the setae from the outer filaments of the compact mantle was clearly evident. These were conspicuous under the dissecting microscope and covered the branches and tips of the forked roots. This was the only specimen in which mycorrhizae with setae were found. Mangin (1910) described much larger setae on *Pinus sylvestris* and other types on *Castanea vesca*. Woodroof (1933) describes different types of setae on pecan and Dominik (1955) illustrates different types of similar structures on spruce, poplar and oak. The author is not aware of previous descriptions on Austrian pine.

Ectendotrophic Mycorrhizae

The most noticeable characteristic of this type as observed in the field was the absence of rhizomorphs, coralloids and macroscopically visible fungus mantles. Similarly, no fungus mats or coralloids were found on the root-ball in greenhouse pots. The typical structure was in the form of single or multiple forked roots, often long "stalked,"

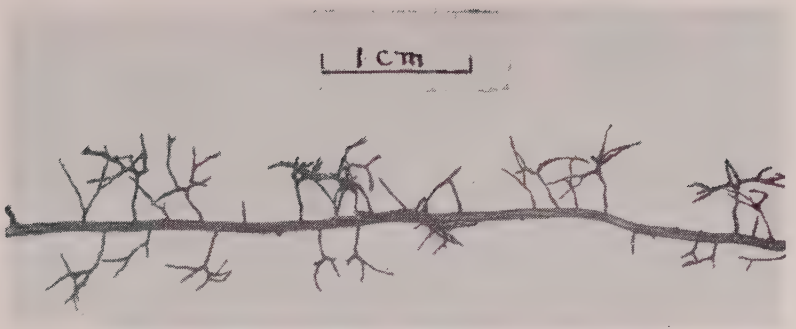

Figure 5. Ectendotrophic mycorrhizae showing many of the characteristics commonly attributed to pseudomycorrhizae (ponderosa pine in inoculated Sand Hill soil).

dark brown, thin, with branches of irregular length and smooth, without any visible mantle under the dissecting microscope (Fig. 5).

On sectioning, these forked roots were found to have a variable but relatively thin mantle about 25 microns thick, however the mantle was often only 10 to 15 microns except in the crotch of the branches where it was always thicker. It was usually composed of 2 to 6 layers of closely packed strands of regularly septate, coarse hyphae 5 to 7 microns in diameter (Fig. 6). There was an extensive intercellular net throughout the cortex appearing as a chain of bulbous cells sometimes up to 10 or 12 microns in diameter. There was some hypertrophy of the cortex but it did not appear to be as great as in the ectotrophic types. A tannin layer was present and in rare instances a few root hairs arising from the epidermal cells were present on the branches of the forked roots.

Intracellular infection was limited to the cells of the cortex and was usually heaviest in the outer two or three cells (Fig. 7). While intracellular hyphae were usually present, the extent of intracellular penetration varied greatly in forked roots which appeared identical in gross characteristics and in type of mycelium, mantle and net. In some sections no intracellular hyphae could be discerned, in others it was absent in the branches of the forked roots and abundant in the "stalk" while in some sections all cortical tissue was extensively invaded. The cellular invasion varied from individual hyphal strands to cells completely filled with hyphae. In some instances the intracellular hyphae became granular and appeared to be in different stages of disintegration. This might be interpreted as digestion of the hyphae by the cell preceeding the formation of the Hartig net as reported in the literature. No definite answer can be made on this point as no special staining techniques were employed to study the cell contents. It should be noted, however, that the intracellular hyphae occurred only in

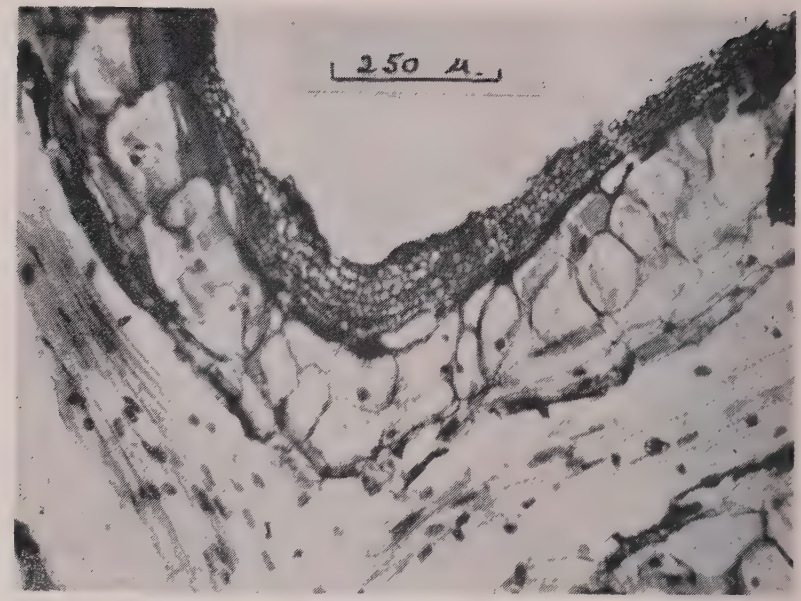

Figure 6. Longitudinal section of ectendotrophic mycorrhiza showing thick compact mantle in crotch of forked root (ponderosa pine).

cells at least partially surrounded by the Hartig net, and the net was extensive in cortical tissue in the absence of any intracellular hyphae. It also was evident that the intracellular hyphae was always more abundant in the older tissues of the short roots, often completely filling the cortical cells which were devoid of normal cell contents. There was no evidence of a diseased condition resulting from such invasion of the old cortex, and the meristematic tissues continued to function.

The gross characteristics of these mycorrhizae are similar to many of the published descriptions of pseudomycorrhizae and they also have some of the characteristics of the haustorial type of infection described by Levisohn (1954) as aberrant root developments. The presence of a mantle and intercellular net, the lack of infection of the stele or meristem and the absence of observed harmful effects would, however, classify them as true mycorrhizae of the ectendotrophic type.

Ectendotrophic mycorrhizae have been reported many times from other countries, but Hacskalo and Palmer (1957-a), working in the United States, noted the ectendotrophic form only once and then on the roots of white pine in a plantation with reduced seedling vigor. Thomas (1943) did not find the ectendotrophic form on pine, including *P. ponderosa*, in his study of the types of mycorrhizae found in Colo-

Figure 7. Longitudinal section of an ectendotrophic mycorrhiza showing coarse intracellular mycelium (ponderosa pine).

rado. On the other hand, McDougal and Jacobs (1927) report the presence of ectendotrophic forms in two collections of *Pinus murrayana* in Colorado at an elevation of 9500-10,000 feet. McComb (1943) describes an ectendotrophic type on white and jack pine seedlings that were unfertilized but inoculated with pine duff. He states that "there was no sharp dividing line betwen the ecto- and ectendotrophic types" and suggests that the same fungus might be involved with different degrees of infection depending on relative vigor of host and fungus. In his fertilized but uninoculated seedlings only the ectotrophic type was found.

Raynor and Nielson-Jones (1944) emphasize the great variability in the manner and incidence of intracellular infection and the importance of the delicate balance, influenced by fluctuating soil factors, between fungus and host. Some workers have considered these ectendotrophic forms to be transition stages between the ectotrophic and endotrophic forms. Hacskaylo and Palmer (1957-a) suggest that they represent "either a pathogenic tendency on the part of an ectotrophic fungus under conditions unfavorable for the higher symbiont or a

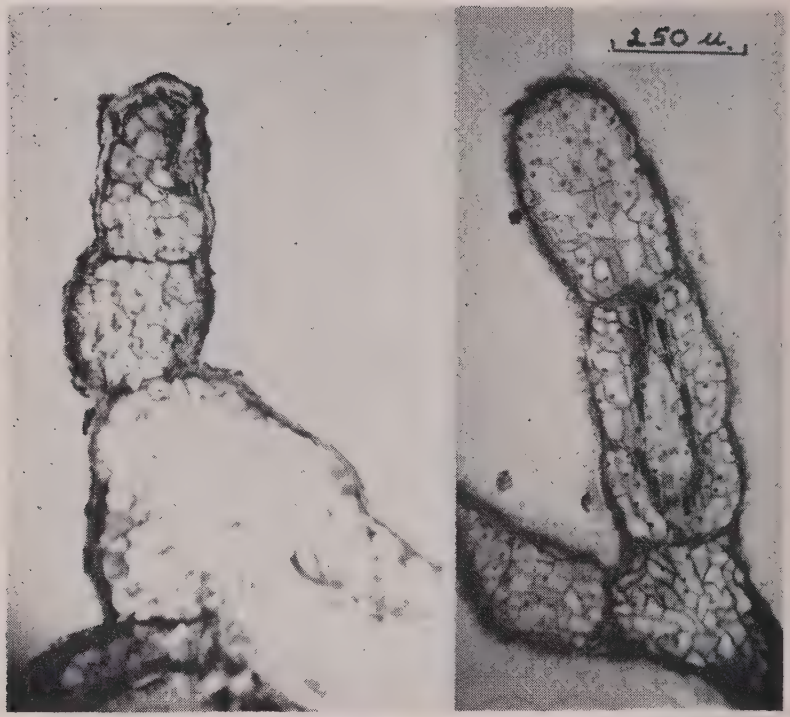

Figure 8. Longitudinal sections of mycorrhizae showing elongation of the forked root. Left, ectendotrophic and right, ectotrophic (ponderosa pine).

secondary invasion by fungi of a weakly parasitic nature brought on by alterations in the rhizosphere."

In the present study, although the fungi involved were not isolated, there was no similarity between the mantle, mycelium and net of the ectendotrophic and ectotrophic types. These ectendotrophic mycorrhizae occurred in the same soils, often on the same root system and occasionally on the same short root as the ectotrophic types.

Elongation of Mycorrhizae

Both the ecto- and ectendotrophic mycorrhizae exhibited many examples of successive elongation and branching of the dichotomously branched short roots (Fig. 8). The branches of these forked roots showed successive constrictions, apparently due to cessation of growth followed by a period of renewed growth. Occasionally, as many as two to four such constrictions were apparent, sometimes with successive dichotomy.When this occurred, the old forked root appeared black and functionless with new tissues being dark brown and each new

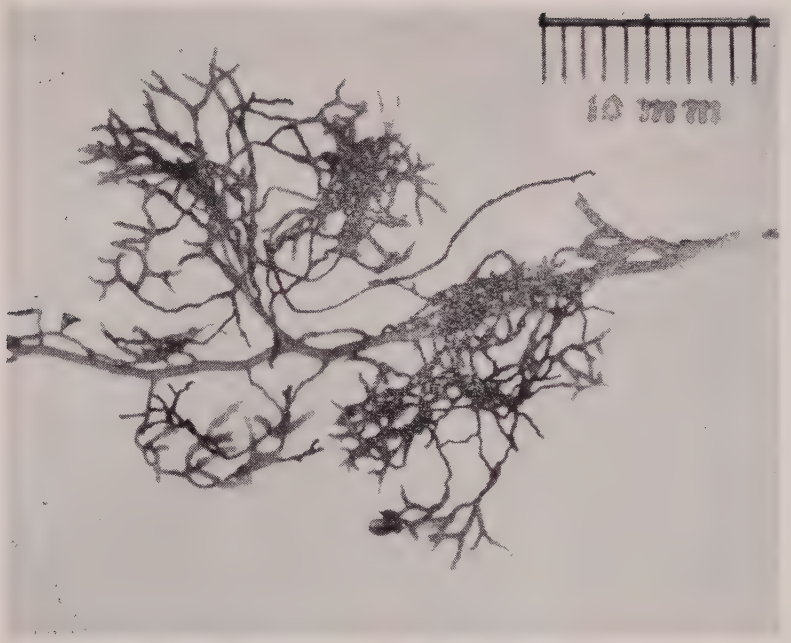

Figure 9. Extensive dichotomy of short roots in absence of fungus associates. Ponderosa pine in non-mycorrhizal Sand Hill soil.

increment of growth being a lighter brown with the youngest being white. The intracellular invasion was usually heaviest just above each constriction with the youngest tissue often showing only intercellular hyphae. Usually, the new growth was of slightly greater diameter than the tissues from which it originated. In some instances the hypertrophy of the cortex was very pronounced, with individual cells having a radial diameter twice as great as the longitudinal, resulting in severe contortion of the entire forked root.

Dichotomous Branching Without Fungus Associates

It is often stated in the literature that dichotomous branching of the short roots of pine rarely occurs in the absence of the fungus symbionts. Hatch and Doak (1933) state that uninfected short roots of pine may exhibit frequent but not profuse dichotomy and that it is rare in nature, occurring chiefly in experimental sand culture.

In specimens examined in Nebraska, dichotomy in the absence of fungus infection was found to be common rather than rare. While single forked roots were most common, multiple forking occurred fairly frequently and, in a few cases, the number of such forked roots would be several hundred within an area of one to two inches. These

groups of forked roots differed from coralloids in the absence of any mantle, the much longer "stalks" and branches, the light brown color and, instead of a compact mass of branched roots, there was a loose tangle of slim forked roots (Fig. 9). The individual "stalks" usually had 4 to 8 forkings and as many as 20 to 30 forked roots might occur on 3 or 4 "stalks" on 5 mm. of a secondary lateral. Under adverse conditions, such as drought, there was evidence of successive elongation much the same as described above for mycorrhizae. While dichotomy is usually considered to be the result of infection with mycorrhizal fungi some sections were found where infection occurred after dichotomy inasmuch as only one branch of the forked root would be affected, with the other branch and the older tissue below the branches showing neither a mantle nor an intercellular net.

Dichotomy without fungus infection occurred on ponderosa seedlings grown in either mycorrhizal or non-mycorrhizal soils and also occurred on mycorrhizal nursery seedlings and forest trees. Other workers have shown that dichotomy may be produced by fungus secretions and by synthetic auxins even to the extent of structures resembling coralloids and that root hairs are absent on such dichotomously branched short roots. In the uninfected forked roots examined in this study, root hairs from epidermal cells were usually present on the branches of the forked roots. It appears that in ponderosa pine at least, dichotomy is an inherent morphological characteristic which becomes more pronounced by the fungus association and results in extensive proliferation and the production of coralloids.

Many of these non-infected forked roots may have the appearance of mycorrhizae on casual observation. They may look more like mycorrhizae than some true mycorrhizae that take on the appearance of pseudomycorrhizae. These non-infected forked roots eventually turn black and may easily be mistaken for pseudomycorrhizae.

Pseudomycorrhizae

One of the most surprising aspects of the present study was the complete absence of any structures that could be termed pseudomycorrhizal. Admittedly, the term has been used in the literature with a variety of descriptions and meanings but usually with the connotation that such structures are pathogenic and harmful. Just as the descriptions have varied so have the reported causal organisms which have been attributed to different and unrelated fungi. Changed physiological relationships between the root and fungi normally mycorrhizal, conversion of short roots into pseudomycorrhizae under conditions unfavorable for mycorrhizae, and conversion of true mycorrhizae under certain conditions into pseudomycorrhizae have all been suggested by other workers as explanations of these pathogenic structures.

In the sections examined, many from roots suspected of being pseudomycorrhizal, none were found containing internal mycelium

of any type without a well developed Hartig net. No infection was ever found in meristematic tissue nor in the endodermis or stele. In some soils producing relatively weak plants and totally lacking mycorrhizae, the microscopic examination of forked roots which were thought might be pseudomycorrhizal failed to reveal a single case that could be termed pathogenic.

The absence of pseudomycorrhizae in these observations does not check with the report of their occurrence on *P. ponderosa* and other pines in Colorado by Thomas (1943), and the previously mentioned descriptions of pseudomycorrhizae in the United States. The results of the present study are in agreement, however, with the conclusion reached by Wilde (1954) that in his studies the beneficial symbiotic organisms were not replaced by harmful pseudomycorrhizae.

The long "stalked" thin, black, irregularly branched ectendotrophic mycorrhizae without macrosopically visible mantles, as previously described and illustrated, could easily be mistakenly identified as pseudomycorrhizae without microscopic examination. The same would be true of some of the forked short roots described which were free of fungus infection. There is need for more extensive studies under a variety of soil and climatic conditions, with different species of pine and at different stages of root development in order to obtain a more complete picture of mycorrhizal structures. It was soon evident in this study that reliance could not be placed on published descriptions of gross characteristics and that microscopic examination of sectioned material was essential. Such examinations failed to detect any specimens as being pseudomycorrhizal.

FIELD OBSERVATIONS

Examinations made in the field at irregular intervals for several years revealed more abundant development of mycorrhizae in the cool, wet weather of fall than in the spring. This was especially noticeable in the fall of 1958 when many active mycorrhizae were found in October in all of the pine species growing in the Nebraska National Forest at Halsey as compared with a scarcity of well developed mycorrhizae at the same sites the following May. Mycorrhizae of pines in various windbreaks in the central part of the state at the same time in May were found with difficulty. The only exception was an abundance of mycorrhizae in one bed of seedlings at the Bessey Nursery at Halsey. The soil temperatures were low, 48-50° F. at 2 to 4 inches depth, at the time the spring observations were made. Although top growth had started with well developed candles there was little active development of short roots. This is in contrast to eastern United States where it is reported that mycorrhizae are well developed before top growth begins in early spring.

There is general agreement in the literature that the formation of mycorrhizae is dependent upon active root development and is, there-

fore, greatest in spring and fall. There is less agreement, however, as to the exact sequence of development in relation to the seasons. MacDougal and Jacobs (1927), studying tree mycorrhizae in the mountains of Colorado, considered that optimum conditions occurred in the latter part of the growing season. McArdle (1932), working with spruce and white pine in Michigan, reported that the maximum development of mycorrhizae occurred from early September to late November and that by spring most of them appeared to be dead. He found few mycorrhizae formed during the spring and summer months. Henry (1933) reported mycorrhizae on some collections every month of the year in Pennsylvania but stated that they were most plentiful in late fall. On the other hand, some workers have reported greater abundance of mycorrhizae in the spring. Thus, Hacskaylo (1957) states that they occur with greatest frequency in the spring, decreasing in the summer and reaching a second peak in early autumn.

It is possible that the low soil temperatures existing in the early spring in Nebraska might delay the development of the fungi and that the top growth would deplete the carbohydrates in the roots, thus further depressing mycorrhizal formation.

The most conspicuous aspect of mycorrhizal development in this study was the repeated evidence of renewed growth of mycorrhizae from what appeared to be old and functionless structures. It has often been stated in the literature that mycorrhizae are rather ephemeral structures. Raynor and Neilson-Jones (1944) state, "In general, the ectotrophic mycorrhizas of pine and other conifers are annual structures functioning for a single growing season and then perishing." They considered Masui's (1926) excellent description of renewed growth as probably exceptional in conifers. McDougal and Jacobs (1927) considered the ectotrophic types to be annual and reported many old dead mycorrhizae in the spring with no new ones observed until after the middle of July. As previously mentioned, McArdle (1932) reported that in the spring the previous year's mycorrhizae appeared to be dead. Conversely, Preston (1943) reports that the mycorrhizal short roots of lodgepole pine were not annual and that in the spring some of the previous year's mycorrhizae had white active tips that had burst or split the fungus sheath.

Observations made in May in Nebraska revealed that in every collection, coralloid mycorrhizae of the previous year were still attached. They were black, often somewhat shriveled, slender, long "stalked" with long branches sometimes of unequal lengths and without any macrosopically visible mantle. The branches of these old, apparently functionless mycorrhizae often showed renewed growth with the tips appearing white or translucent. This evidence of renewed elongation was common and was found even on those roots which were forming new, short, typical coralloids on the same lateral. Sometimes these old mycorrhizae appeared to have gone through two or three such periods

of successive elongation as described on page 14. These observations, made in the spring just as the new white tips were appearing. were clearly evidence that these constrictions and subsequent elongations of the branches of the forked roots had occurred the previous year. This was borne out by observations made during the summer when such a sequence of growth periods could be observed. At first thought, this might be considered the result of varying conditions of temperature and moisture. This was ruled out, however, by the fact that a similar sequence of development occurred on seedlings in the greenhouse (see p. 25). The phenomenon may be related to the particular fungi involved, which have not been identified or it may be a characteristic of ponderosa pine, a species that has not been studied extensively for mycorrhizae. Another common occurrence was the emergence of new short roots from the lateral and immediately adjacent to the old black mycorrhizae. These were often of the smooth brown type of mycorrhizae without any macroscopically visible fungus mantle.

A less common example of renewed growth of old mycorrhizae was the occurrence of new ectotrophic mycorrhizae with white mantles and rhizomorphs arising from the basal portions of the old black mycorrhizae similar to the description by Kelley (1950, Fig. 4) of such growth on *Pinus strobus*. Sometimes, along with this development, there was also renewed growth of the branches of the old mycorrhizae as described above. Only one example was found of the splitting of the fungus mantle and emergence of the new tip as described by Kelly (1950, Fig. 8 on *P. virginianae*), by Masui (1926) on *P. densiflora* and fir and by Preston (1943) on lodgepole pine.

In considering these observations in Nebraska it must be remembered that the soils and climatic conditions are quite different from those in the forested areas of eastern United States. The sandy soils on which many of these observations were made were not as acid as is usually considered favorable for mycorrhizal fungi. The pH varied from 5.8 to 7.1 which is typical of many of the Great Plains nurseries. Some of these nurseries report even higher pH's. It may be that in such soils and with relatively low rainfall the mycorrhizal fungi are different species than those commonly reported in the eastern states.

Mycorrhizae were found in Nebraska at lower levels in the soil, particularly in the spring, than are commonly reported. In many locations mycorrhizae were found only at depths of 9 to 12 inches. While mycorrhizae have been reported at lower levels in the literature, their occurrence in native stands has usually been found to be in the decaying litter and upper layers of soil.

In one windbreak in which the silt loam soil was compacted by pasturing, the few mycorrhizae found in the spring were not only at a depth of 6 to 12 inches but were also produced in only one plane parallel to the soil surface. Mangin (1910) reported distichous mycor-

rhizae in compressed leaf mold and Woodroof (1933) describes fan-like clusters of pecan mycorrhizae in the heavy red subsoils of northern Georgia.

In brief, the chief characteristics noted in these observations were: the relative sparse production of mycorrhizae; their occurrence in greater abundance in the fall; their late development in the spring in relation to top growth; their occurrence at some depth in the soil; the persistence of the previous year's mycorrhizae; their renewed growth from the tips of the old, black, seemingly functionless branches of the forked roots; the repeated elongation of the mycorrhizal roots; the common occurrence of ectendotrophic forms and the absence of pseudomycorrhizae.

GREENHOUSE EXPERIMENTS

Methods

It has often been stated that it is difficult to conduct mycorrhizal studies in the greenhouse because of uncontrolled contamination by mycorrhizal fungi. Theoretically at least, it should be easier to conduct such experiments with fungi not normally fructifying in the greenhouse than with the many spore forming fungi which the pathologist usually studies in greenhouse experiments. Such pathological investigations, however, are usually for short periods of time as compared with the experiments here reported which usually ran from 6 to 18 months.

Efforts were made in preliminary tests to devise methods that would be satisfactory for these long periods and at the same time provide for uniform stands, sufficient numbers, separation of experimental units while maintaining uniform environmental conditions, economy of space and the minimum of labor. While the methods varied somewhat in each experiment, in an effort to avoid repetition and conserve space, the general methods employed are summarized as follows.

Seed

Because of its importance in this area only one species, *P. ponderosa,* was used. In the early experiments the seed was obtained from a commercial nursery.[1] In all the later experiments the seed was obtained in 2 lots from the U. S. Forest Service and was the same as that being used at the Bessey Nursery at Halsey, Nebraska. One lot had been collected in the Black Hills in 1954 and the other in 1955. In only one experiment was it necessary to use both lots and then the same lot was used for comparable portions of the test.

All seeds were disinfected in 0.1 per cent $HgCl_2$ for 5 minutes and washed in tap water. To obtain uniform stands without overplanting and thinning, all seeds were germinated in sterilized Terra Lite and when the radicle was approximately 2 inches long with the testa still

[1] Courtesy of the Plumfield Nursery, Fremont, Nebraska.

adhering to the cotyledons, straight, erect seedlings were easily pulled and transferred to a prepared hole in the soil under test. If damping-off or root-rot occurred during the first few weeks of an experiment, a re-plant could be easily made to obtain the desired population.

Soil

In all cases the soils were from virgin grassland as far removed as practical from any stand of trees, particularly conifers. In the first tests the soils were from local sites around Lincoln. The one indicated in Table 2 as "grassland" was selected as being virgin grassland comparable to the soil on which a mixed stand of pine had been planted 30 years before. It was located about 250 feet from this stand. The one indicated as "grassland" in Table 3 was from an uncultivated strip of virgin grassland bordering experimental crop plantings and was about one-half mile from a few isolated deciduous trees. The other grassland soils were selected as being representative of three different areas of the state. The analyses of these three grassland soils along with samples of nursery soil and the pine duff-soil mixture are given in Table 1.

The soil designated as "Prairie" was obtained in Gage County as being representative of much of the native grassland of eastern Nebraska. This had been undisturbed except for annual mowing during the last 40 years at least and was in an area with an average annual rainfall of 30 inches. The soil was non-calcareous, dark brown and is classified as a Sharpsburg silty clay loam. There was a thin stand of cottonwoods and elms about one-fourth mile distant but there were no stands of conifers in the vicinity.

The "Sandhill" soil was typical of the extensive Sandhill range land of central Nebraska which, aside from a few stands along the rivers, is devoid of trees. The soil was taken from a site 0.7 mile from the nearest trees and 2 miles from the Nebraska National Forest which has a rainfall of about 20 inches a year.

The soil designated as "Chestnut" was obtained in Box Butte Coun-

Table 1. Some physical and chemical characteristics of the soils used in the greenhouse experiments.[1]

Soil Samples	Particle size analysis, (Hydrometer method) percentage			Percentage moisture content at atmosphere		Percentage of total		pH	Available	
	clay $< 2\ \mu$	silt 2-50 μ	Sand 50–2000 μ	15	1/3	N	organic matter		P. ppm.	K. ppm.
Sandhill	4	4	92	2.6	5.1	0.050	1.3	6.1	< 3	110
Chestnut	18	29	53	10.0	20.0	0.179	2.9	6.8	26	550
Prairie	26	51	23	14.0	32.0	0.234	3.1	5.8	< 3	180
Nursery	12	26	62	4.0	7.0	0.055	1.5	7.1	>30	153
Duff-soil mixture	5	5	90	4.0	6.0	0.071	2.1	6.0	8	143

[1] The author is indebted to the Agronomy Department of the University of Nebraska for the analytical data in the Table.

ty in western Nebraska and was considered typical of the grassland found on these treeless tablelands of the western part of the state at an elevation of about 4,000 feet and with an annual rainfall of about 15 inches. The site was ¾ mile from a single row of pines and about 50 miles from any forested area. The soil is classified as Rosebud fine sandy loam.

All of the grassland soils were taken from the top 4 to 6 inches of sod and included many grass roots. These were largely removed by the use of a ¼-inch mesh screen. After screening the soils were stored in 20-gal. galvanized containers and as some of the samples were very dry when collected, the soils were moistened before storage.

The duff-soil mixture used as inoculum was obtained by removing the top layers of undecayed litter and taking the next four or five inches of decaying litter and mineral soil from the A_0 and A_1 horizons. This contained many mycorrhizal pine roots. The mixture was screened and stored in the same manner as the soils listed above. In the first experiments the sample was collected from a mixed stand of Austrian and ponderosa pine from Pioneers Park, Lincoln, which had originally been planted with nursery grown stock. In later experiments the duff-soil mixture was collected from a 50-year-old stand of ponderosa pine planted in the Nebraska National Forest at Halsey located in the sandhill area previously referred to. Its analysis is given in Table 1.

The nursery soils were either from the Bessey Nursery at Halsey or from a commercial nursery (Table 1) in eastern Nebraska. The soil samples in each instance were taken from the top 4 to 5 inches of beds of pine seedlings.

Soil Treatments

Inoculum.—In the early experiments the duff-soil mixture was added to the grassland soils at the rate of one percent by weight and in the later experiments at the rate of five percent.

Sterilization.—In some of the tests the soil was sterilized either by flowing steam on three successive days or by autoclaving for 2½ hours at 15 lbs. pressure.

Fumigation.—In order to avoid the physical and chemical changes due to steam sterilization, fumigation was used in some experiments. Chloropicrin was used in one experiment (Table 2). In the later experiments methyl bromide (Dow Fume Mc2) was used as this is a very effective fumigant for the elimination of mycorrhizal fungi. It was used at an excess rate and covered for 48 hours after which the soil was aerated for 10 days before planting.

Containers.—To conserve space and ensure uniformity of soil environment the first tests were carried out in wooden flats holding 25 seedlings each. This practice was discontinued because of the difficulty of examining the individual root systems and the danger of a single contamination affecting the results with all 25 seedlings. Four-

inch clay pots with one seedling per pot were found to be ideal in permitting periodic inspection of the root-ball but the amount of soil was insufficient for 12 to 18 months' continuous growth and this method was not used in the later experiments.

The method eventually employed in most tests was the use of 7-inch clay pots with 3 seedlings per pot. The hole in the pot was plugged with glass wool and the pots were sunk into peat moss. Each set of pots was isolated by partitions of galvanized steel with about four inches of open bench between the partitions, sufficient to prevent drainage water from becoming a hazard. Watering was necessary only once a week.

Light.—Length of day and light intensity have been reported to influence the development of mycorrhizae. Daylight was therefore supplemented by 100-watt lights at 4 ft. intervals and for periods varying with the day lengths to give a total of 21 to 24 hours of light each day.

Temperature.—All tests, unless otherwise noted, were carried out in a greenhouse maintained at 70-80°F. During summer months the pots were transferred to a greenhouse where this temperature was maintained by refrigeration.

Final Notes and Measurements.—The height of seedlings was determined by measuring in millimeters from the cotyledons to the terminal bud. The diameter of the collar was measured to the nearest 0.5 millimeter. The green weight of tops was determined in grams. In the absence of coralloid mycorrhizae determinations were based on stained sections. This provided a more accurate appraisal of the occurrence of mycorrhizae than the presence of fungus mats, rhizomorphs and dichotomously branched short roots which were also recorded.

Fixing, Sectioning and Staining.—Specimens of roots selected for microscopical study were washed in water and preserved in formalin-acetic-alcohol. Sectioning was done with a freezing microtome cutting at 20 microns and the routine stain employed was Staughton's Thionin and Orange G.

Sequence of Mycorrhizal Development in Seedlings

The field observations reported were chiefly on mature trees and although nursery seedlings were also included, no attempt was made to study the sequence of mycorrhizal development.

It appeared desirable to undertake a preliminary study of the development of seedlings in a mycorrhizal soil under uniform conditions as an aid to the interpretation of the results of greenhouse experiments. Ponderosa pine seeds were germinated in Terra-Lite and transplanted into pots of the duff-soil mixture from the 50-year-old stand of ponderosa in the Nebraska National Forest at Halsey. Five seedlings were grown in each 6-inch clay pot in the greenhouse at 70°

to 80° F. for a period of 10 months. At the time of transplanting, the testa were still attached to the cotyledons and the radicles were 4 to 6 cm. in length. At intervals the seedlings in one pot were removed, examined for mycorrhizae, and the roots were fixed for sectioning and staining.

There were no dichotomously branched short roots at the end of two months but numerous simple short roots on the 5 to 12 laterals on each seedling. Some of these short roots were thickened, lacked root hairs and were found to be ectendotrophic mycorrhizae. There was a thin smooth mantle of coarse mycelium, 5 to 6 microns in diameter, a well developed intercellular net and some intracellular infections in the basal cells of the cortex which was hypertrophied. Both inter- and intracellular mycelium were also present in the lateral root in the vicinity of the infected short roots and appeared continuous with that in the short root. There was no hypertrophy of the cortex of the lateral and no mantle although there were individual strands of mycelium on the surface similar to the internal mycelium. At this stage the tap roots were 210 to 430 cm. long with numerous lateral mother roots.

Dichotomously branched short roots were present when examined 111 days after transplanting and the number varied greatly on the individual seedlings. Only the smooth brown ectendotrophic type of mycorrhizae were found. The compact mantles of these were now well developed, sometimes up to 50 microns thick, and the intracellular infection was extensive.

The first coralloid ectotrophic mycorrhizae were found at the next inspection 42 days later, 153 days after transplanting. Only one was found on only one of the five seedlings. This had a white fungus mantle composed of fine mycelium 2.5 microns in diameter and with clamp connections. The mantle was about 35 microns thick and there was a well developed Hartig net and no intracellular infection. The numerous ectendotrophic types were now showing successive dichotomy with 6 to 8 branches on "stalks" usually 3-5 mm. long but occasionally up to 10 mm. The lateral which produced the ectotrophic mycorrhizae also had numerous smooth brown forked short roots of the ectendotrophic type. When the lateral was sectioned both inter- and intracellular mycelium were found from a point 2 mm. back of the meristem of the root tip to the base of this 80 mm. long lateral.

The seedlings examined after 196 days were devoid of ectotrophic mycorrhizae but had developed numerous ectendotrophic forked roots with successive elongations. The "stalks" were often dark brown to black with the basal portions of the branches being dark brown; then a constriction and renewed growth, light brown in color and of increased diameter; followed by another constriction and a white tip of new growth.

At the next examination, 247 days after transplanting, there were fungus mats with many rhizomorphs and numerous coralloid ectotrophic mycorrhizae with white fungus mantles. The numerous smooth forked roots of the ectendotrophic types varied from new light brown ones to old black ones showing as many as four constrictions with successive stages of growth. The tap roots were 3 to 5 feet in length.

In later examinations made during the 10-month period the ectotrophic mycorrhizae were often in the form of massive coralloids almost nodular in appearance and covered with dense white fungus mantles. The ectendotrophic types continued to show successive stages of elongation. Most of the new short root development during this period appeared to be in the elongation and successive branching of the short roots rather than in the development of new short roots on the laterals.

It was evident from these observations that the duff-soil mixture obtained from a 50-year-old healthy stand of ponderosa pine in Nebraska contained both the basidiomycetous ectotrophic mycorrhizal fungus and the unidentified fungus with coarse mycelium constantly associated with ectendotrophic mycorrhizae. The latter type appeared to be more prevalent at least in the early stages of growth with extensive infections prior to any evidence of dichotomy and long before the appearance of fungus mats, rhizomorphs and coralloid mycorrhizae with white fungus mantles characteristic of the ectotrophic types. Only the ectendotrophic type of mycelium was found in the laterals even in those which also produced ectotrophic mycorrhizae.

The successive elongations of the forked short roots were similar to those observed in the field both in nursery stock and mature trees. In this instance, however, such evidence of renewed growth of mycorrhizae could not be attributed to seasonal factors but could be influenced by similar metabolic changes in the growth of the seedling even though growing under fairly constant environmental conditions.

Pure Culture Inoculations

During this study a large number of isolations were attempted from mycorrhizal roots and rhizomorphs using a variety of methods described in the literature as being satisfactory. Many of the isolates produced only sterile mycelium but none of them had clamp connections such as occurred in the mycelium on some of the roots from which the isolations were made. Those which were isolated most consistently along with cultures of *Boletus felleus* and *Cenococcum graniforme*[1] were used to inoculate *P. ponderosa* seedlings. No inoculations were attempted with aseptic cultures of seedlings. Open culture in pots in the greenhouse was resorted to with dependence being placed on uninoculated controls. Unfortunately, no culture medium

[1] Cultures of these species were obtained through the courtesy of Edward Hacskaylo, U. S. Forest Service.

was found that proved satisfactory for both seedling and fungus as judged by the lack of mycorrhizae.

A total of six different tests of 26 different cultures of suspected fungi were made on 185 seedlings with 40 uninoculated controls. The reliance placed on the controls was well founded as mycorrhizae were found in only one of the controls. However, only one pot of inoculated seedlings produced mycorrhizae and none resulted from the repeated tests with *B. felleus* and *C. graniforme*.

Owing to these negative preliminary tests and lack of time for a more comprehensive investigation, this phase of the study was discontinued and gross inoculation with duff-soil mixture was resorted to in the following experiments.

Occurrence or Establishment of Mycorrhizae in Different Soils

In a preliminary experiment, ponderosa pine seedlings were grown in greenhouse flats of different soils with 25 seedlings per flat. After seven month's growth the occurrence of mycorrhizae and the vigor of the seedlings were recorded (Table 2).

There was a marked contrast between the very vigorous growth in the duff-soil mixture and grassland soils and the weak seedlings in the cultivated field and nursery soils. There was no increased growth in the inoculated grassland soil over the addition of sterilized or fumigated inoculum. The weak condition of the seedlings growing in cultivated field soil was not alleviated by the addition of inoculum.

All of the seedlings in the grassland soils had coralloid mycorrhizae and both ecto- and ectendotrophic types were present in the untreated grassland soil. It appeared that these were not due to the addition of inoculum as no coralloid mycorrhizae appeared on the seedlings growing in the duff-soil inoculum. These roots, however, were found to have both types of ectotrophic mycorrhizae and also ectendotrophic mycorrhizae.

The cultivated field soil had some single forked short roots but

Table 2. Relation of soil to mycorrhizae and vigor of pine seedlings.

Soils and Treatments	Relative vigor of seedlings	Green weight of tops (grams)	Relative abundance of coralloid mycorrhizae	Occurrence and type of mycorrhizae 2/	
				Ecto-	Ectendo-
Grassland[1]	++++	3.0	+++	+	+
Grassland, inoculated	++++	3.3	+	+	0
Grassland + sterilized inoculum	+++	3.5	+	+	0
Grassland + fumigated inoculum	++++	3.2	+	+	0
Cultivated field soil	+	2.1	0	0	0
Cultivated field soil, inoculated	+	2.6	0	+	0
Nursery soil	++	2.7	0	0	+
Duff-soil mixture inoculum	+++++	4.1	0	+	+

[1] The grassland soil was from Pioneers Park, Lincoln, 250 feet from a mixed stand of pine from which the inoculum was obtained. Sterilization was in flowing steam for one hour on three successive days and fumigation was with chloropicrin.

[2] Type of mycorrhizae determined by microscopic examination.

no trace of fungus infection could be found. Inoculation of this soil failed to produce any coralloid mycorrhizae but sections of the single forked roots revealed ectotrophic mycorrhizae although these roots appeared indistinguishable from those without infection. The roots in the nursery soil failed to produce coralloid mycorrhizae or forked roots with macroscopically visible mantles but the stained sections showed the ectendotrophic mycorrhizae.

The abundance of mycorrhizae in the grassland soil could hardly be attributed to contamination in the greenhouse when the other soils, particularly the duff-soil mixture, failed to produce such coralloid mycorrhizae. It is possible that this particular grassland soil, located only 250 feet from a mixed stand of pine, had become infested with wind-blown spores of fruiting hymenomycetes (*Boletus, Lycoperdon* and *Tricholoma* spp.) which were present in the pine stand at the time the soil samples were taken in October. This appeared even more probable when it was noted that ectotrophic Type 1 with fine mycelium often having clamp connections was the predominant type in these roots as it was in the duff-soil mixture. The lack of coralloid mycorrhizae in the roots growing in the duff-soil mixture is not explainable by the data in this test as the same mixture in another test (Table 3) produced many coralloids.

Another experiment essentially similar to the previous one was made but with a different source of grassland soil and with the seedlings grown in individual 4-inch pots instead of in flats. The seedlings were grown for approximately 15 months and during the summer the pots were transferred outdoors to a lath-house where the temperatures were higher than desirable, occasionally going above 90°F. The final data are presented in Table 3.

Contrary to the results of the previous experiment the grassland soil used in this test (Set 1) did not contain mycorrhizal fungi as judged by the freedom of 7 of the 10 seedlings from any fungus mats,

Table 3. Response of ponderosa pine seedlings and mycorrhizae to various soils and treatments.

Set No. (10 plants each)	Soil and Treatment[1]	Aver. Ht.	Aver. green top weight	Aver. diam. of collar	Mycorrhizal seedlings[2]
		mm.	grams	mm.	No.
1	Grassland	123	14.2	4.1	3
2	Grassland, inoculated	141	19.5	4.2	10
3	Grassland + sand	120	14.2	3.8	2
4	Grassland + sand, inoculated	181	18.4	4.4	9
5	Nursery soil No. 1	120	12.2	3.5	4
6	Nursery soil No. 2	100	8.5	3.3	4
7	Duff-soil mixture inoculum	132	15.5	4.0	10

[1] The grassland soil was from University of Nebraska Agronomy Farm, the inoculum from Pioneers Park, Lincoln, Nursery Soil No. 1 from a commercial nursery and No. 2 from Bessey Nursery, U. S. Forest Service. See page 21 for details.

[2] All mycorrhizal seedlings had coralloids except two in Set 1 which had only light fungus mats and rhizomorphs and with forked roots which were determined by sectioning to be mycorrhizal.

rhizomorphs or microscopic evidence of mycorrhiza even though there were single and multiple forked short roots. Ectotrophic mycorrhizae of Type 1 were present in the other three seedlings and were probably due to contamination. In Set 3 of the same soil plus sand, two of the seedlings were apparently contaminated, the forked roots had sparce white fungus mantles but no rhizomorphs and there were no coralloids. Both were ectotrophic, one with both Type 1 and 2 mantles and the other with Type 2. The other eight seedlings had occasional uninfected forked roots often with long "stalks" and branches of unequal length.

In contrast to these results with grassland soil the same soil when inoculated (Sets 2 and 4) produced fungus mats, rhizomorphs and coralloid mycorrhizae. These proved to be ectotrophic Type 1 mycorrhizae but some showed the presence of ectendotrophic mycelium at the base of the "stalk" of the forked root with both inter-and intracellular infection and with one or two strands of the coarse mycelium under the mantle of fine mycelium. The duff-soil mixture (Set 7) also produced ectotrophic Type 1 coralloid mycorrhizae in every seedling.

Both nursery soils produced a few coralloid mycorrhizae but not so abundantly as the inoculum or the inoculated grassland soil. Fungus mantles and rhizomorphs were sparse or absent and both ectotrophic and ectendotrophic mycorrhizae were present.

The quantitative data in Table 3 must be viewed with some reservation as there was some escape of roots into the peat moss. Nevertheless, the tallest, heaviest and sturdiest seedlings were growing in inoculated grassland soil. On the other hand, there was no significant difference between the uninoculated grassland soil (Sets 1 and 3) and the duff-soil mixture in Set 7. Apparently the higher fertility of the grassland soil offset the effects of mycorrhizae. The nursery soils which were the lowest in fertility produced the weakest plants even though mycorrhizae occurred. It has generally been considered that formation of mycorrhizae and their beneficial effects are greatest in soils of low fertility but there was no evidence to support this view in this experiment.

It was apparent that the duff-soil mixture from a 35-year-old stand of mixed pine planted on virgin prairie sod with nursery grown stock contained mycorrhizal fungi. The virgin grassland soil used in this test did not contain such fungi but permitted their growth and the formation of mycorrhizae when inoculated at the time of planting.

In another experiment with the same grassland soil, inoculum and nursery soil, the results were quite similar. After one year's growth in 4-inch pots with 10 pots for each soil, the seedlings averaged as follows:

Grassland soil	height	142 mm.	collar diameter	3.8
Grassland inoculated	"	164 mm.	" "	4.3
Duff-soil inoculum	"	134 mm.	" "	4.3
Nursery soil	"	100 mm.	" "	3.4

There were fungus mats and mycorrhizae on the root-balls of all of those in duff-soil inoculum or in inoculated grassland soil but none were present in the nursery soil at the end of one year although eight months later all 10 in nursery soil had coralloid mycorrhizae. Seedlings in two of the pots in grassland soil had mycorrhizae, probably due to contamination.

After one year eight of the ten pots of grassland soil without mycorrhizae had many dichotomously branched short roots without any evidence of mantles or fungus invasion. There was no hypertrophy of the cortex, and epidermal roots hairs occurred on the branches of the forked roots.

All of the seedlings in duff-soil inoculum or in inoculated grassland soil produced coralloid mycorrhizae with many rhizomorphs. The mycorrhizae were of the ectotrophic Type 1 with fine mycelium. In some roots the ectendotrophic type was present in the older tissue of the "stalk" of the forked roots with the ectotrophic form in the branches. Some of these forked roots showing both types of infection were long "stalked" (6 mm.), black, slender and with unequal branches 2 to 3 mm. long, similar to some of the descriptions of pseudomycorrhizae or aberrant types. The microscopic characteristics of these forked roots, however, were as described (p. 10) for ectendotrophic mycorrhizae.

Three different virgin grassland soils were then tested as representative of three soil types from widely separated areas of the state. These were: a clay loam Prairie soil from eastern Nebraska, a Sandhill soil from the extensive Sandhill region of the north central area of the state and a Chestnut soil from the table-land area of western Nebraska. The duff-soil mixture used as inoculum was from the 50-year-old stand of ponderosa pine in the Nebraska National Forest. For comparison one set of seedlings was grown in soil from a commercial nursery.

The three grassland soils varied greatly in physical and chemical analysis as seen in Table 1. The Prairie soil had the lowest content of sand and was the highest in N and organic matter and had the lowest pH (5.8). The Sandhill soil was almost pure sand and was very low in N and organic matter with a pH of 6.1. The Chestnut soil was midway between these two in content of sand, was nearly as high as the Prairie soil in N and organic matter, had the highest pH (6.8) and was very high in available potassium.

The seedlings were grown for 14 months and then removed from the pots and final notes recorded. In the three uninoculated grassland soils the greatest growth as determined by height, collar diameter and green weight occurred in the richer Prairie soil (Table 4, No. 3), the least growth was in the Sandhill soil (No. 1) with the Chestnut soil (No. 2) midway between the two.

The inoculation of these soils resulted in increased height and

Table 4. Effect of soil and inoculation on the development and survival of ponderosa pine seedlings.

Series No.	Soil	Seedlings					
		Planted	Chlorotic[1]	Survived	Ht.	Collar diam.	Green wt.
		No.	No.	No.	mm.	mm.	gm.
1	Sandhill	30	4—	30[2]	91	3.3	11
1-D	Sandhill, inoculated	24	0	24	109	3.3	14
2	Chestnut	30	24+	23[2]	100	3.9	16
2-D	Chestnut, inoculated	24	1—	22	133	3.8	19
3	Prairie	31	9—	31[2]	121	3.9	17
3-D	Prairie, inoculated	24	0	24	149	4.1	22
4	Commercial nursery soil	24	7±	24	75	3.2	9
5	Duff-soil mixture inoclum	24	0	24	112	3.5	16

[1] Degree of severity: — slight, ± moderate, + severe.
[2] Two pots in soil No. 1, and one each in soils 2 and 3 had become definitely mycorrhizal and therefore are not included in the measurements.

weight, but had no appreciable effect on the collar diameter. The effect of inoculation as determined by percentage increase in height and weight respectively was: Soil No. 1—20 and 27 percent; Soil No. 2—33 and 18 percent; and for Soil No. 3—23 and 30 percent. This increase from inoculation was obvious with even a casual observation.

The poorest growth in the experiment occurred in the nursery soil (No. 4) which was about the same as the Sandhill soil in available N and with a high pH of 7.1. The duff-soil inoculum (No. 5) was about the same as the Sandhill soil (No. 1) in physical structure, N, pH and available P and K, but produced more vigorous seedlings than the Sandhill soil. As might be expected there was very little difference between the seedlings grown in duff-soil inoculum and those in the inoculated Sandhill soil. The growth was not as great as the more fertile soils which had been inoculated (2D and 3D).

The most noticeable phenomenon in this experiment was the unexpected occurrence of chlorosis which was most severe in the Chestnut soil (No. 2). The first symptoms of chorosis appeared on a few plants soon after the secondary needles were formed. The symptoms became progressively more severe although some of the seedlings continued to survive for many months even after all the needles became a bright yellow and terminal growth ceased. At the end of 8 months two of the seedlings that had shown early symptoms were dead and 17 others out of the original 30 were chlorotic in varying degrees. By the end of the experiment 7 had died of chlorosis, 5 were severely affected with many of the needles having turned brown and dropped, 7 were moderately and 6 slightly affected. Only 5 seedlings remained healthy and three of these were in one pot and the roots of these on microscopic examination were found to be mycorrhizal.

Contrasted with this the seedlings in the same Chestnut soil to which 5 percent of inoculum had been added showed very little

Figure 10. Effect of mycorrhizae on chlorosis. Twelve-months-old ponderosa seedlings in six inch pots of (left) inoculated Chestnut grassland soil, (right) same soil uninoculated.

chlorosis. After 8 months only 4 seedlings out of 24 showed any symptoms and these were very slight. Three of these four recovered and had healthy green foliage at the end of the experiment. Only one seedling continued to exhibit very slight symptoms. This recovery was just the opposite of the same soil (No. 2) without inoculation in which the disease became progressively worse throughout the experiment.

The data on height and weight presented in Table 4 are in agreement with the observations made on plant vigor throughout the experiment (Fig. 10). It must be remembered that seven of the 30 plants in the uninoculated Chestnut soil died as a result of severe chlorosis and quantitative data based on the original number of seedlings would show much greater differences.

Examination of the root-balls at the end of the experiment revealed that fungus mats were present with coralloid mycorrhizae in 4 of the 8 pots in Soil 2-D and the roots in only 1 of the 8 failed to show mycorrhizal short roots. None of the root-balls in the 10 pots of uninoculated soil (No. 2) had fungus mats or coralloid mycorrhizae, but the roots in the pot containing three healthy seedlings had numerous single and multiple dichotomously branched short roots and were found to be type 2 ectotrophic mycorrhizae. Apparently this one pot had become contaminated and the effect was the same as in soil 2-D which was inoculated. Microscopic examination of a random selection of single dichotomously branched roots from the other 9 pots of Soil 2 failed to reveal the presence of any fungus either internally or as a mantle.

Table 4 shows that this same effect of inoculation on chlorosis is evident but to a lesser extent with the other two grassland soils tested.

The Sandhill soil (No. 1) had four slightly chlorotic seedlings while none occurred when inoculum had been added (1-B). Similarly the Prairie soil (No. 3) had 9 slightly chlorotic plants whereas none occurred when inoculated (3-D).

This beneficial effect of the addition of a small amount of duff-soil inoculum to a virgin grassland soil in preventing chlorosis is strongly indicative, but does not definitely prove, that the mycorrhizal fungi introduced were the causative factors. The chemical analysis in Table 1 shows that the inoculum (No. 5) which was added had a lower nutrient content than the grassland soil (No. 2). A further test would be needed, however, to determine whether or not the addition of some minor element was involved.

Most of the pots containing duff-soil inoculum or inoculated grassland soil had root-balls with white fungus mats, rhizomorphs and coralloid mycorrhizae (Figs. 1, 2). These were ectotrophic Type 1 mycorrhizae and showed successive elongation of the branches of the forked roots. Only two specimens examined had the Type 2 ectotrophic mycorrhizae.

Ectendotrophic mycorrhizae were found only in the commercial nursery soil and in the one contaminated pot of soil No. 2. When both types of mycorrhizae were present the fine mycelium of the ectotrophic type invariably was present in the branches and the coarse mycelium of the ectendotrophic type was in the "stalk" of the forked root and at the base of the branches.

Another experiment using only the Prairie and Sandhill soils, differed from the previous test by using methyl bromide fumigation prior to planting to eliminate any mycorrhizal fungi which might be present in the soils. In addition, both fumigated and unfumigated inoculum were tested to determine whether the addition of the duff-soil inoculum would have an effect on the development of seedlings not attributable to mycorrhizal fungi.

Seedlings were grown for 14½ months under conditions similar to the previous tests but, unfortunately, with an air temperature which often reached a maximum of 90° to 100° F. in July and August. Soil temperatures in the morning averaged in the 70's with no temperature being recorded above 80° F. In the afternoons, however, the soil temperatures recorded were in the 80's in June, about 90° in July and as high as 90° to 98° F. on many days in August.

As in the previous test some chlorosis occurred and was more severe in the Prairie soil than in the Sandhill soil (Table 5). Unfortunately the Chestnut soil which produced such severe chlorosis in the previous experiment was not included in this test but the effect of inoculum was evident in the Prairie soil. This soil had fewer chlorotic seedlings when untreated inoculum was added than where fumigated inoculum was used. In this latter soil (1B, Table 5) chlorosis appeared early with six of the ten pots having one or more seedlings showing chlorosis

Table 5. Response of ponderosa pine seedlings to the inoculation of fumigated grassland soils.

Series No.	Soil and Treatments[1]	Planted	Damping-off and root rot	Chlorotic	Surviving plants	Ht.	Diam. collar	Green wt. tops
		No.	No.	No.	No.	mm.	mm.	gm.
1A	Prairie, inoculated	24	10	2	21	98	3.8	11
1B	Prairie + fumigated inoculum	30	2	11	27	90	3.7	10
2A	Sandhill, inoculated	24	4	2	24	113	4.2	17
2B	Sandhill+fumigated inoculum	30	2	2	30	114	4.7	16
3A	Duff-soil mixture inoculum	12	3	0	12	102	4.1	18
3B	Fumigated inoculum	15	3	0	15	122	3.8	14

[1] All soils except 3A were fumigated with methyl bromide before inoculation. No mycorrhizae occurred in soils to which fumigated inoculum was added.

in the juvenile needles. Three seedlings died of chlorosis. Apparently the beneficial effect of inoculum was due to its biological action as the effect was lost upon fumigation with methyl bromide which is very effective against mycorrhizal fungi. No effect of inoculation on chlorosis could be detected in the Sandhill soil, which had a low incidence of the disease. No chlorosis occurred in the duff-soil inoculum either untreated or fumigated.

Other than the data on chlorosis, the results of this experiment do not agree with those obtained in the previous experiment. The Sandhill soil produced much larger and sturdier plants than the Prairie soil, the reverse of the previous test. Except with respect to chlorosis in Soil 1, there was also no confirmation of the beneficial effects of inoculation on the growth of the seedlings, although this effect was consistent and conspicuous in the previous test.

There were no fungus mats nor coralloid mycorrhizae in any of the pots of inoculated Prairie soil (1A). There were numerous single and double forked short roots which on sectioning were found to be free of fungus infection. Similar dichotomously branched short roots occurred in the Prairie soil (1B) to which fumigated inoculum had been added.

There was also a lack of any beneficial effect due to inoculation of the Sandhill soil. Unlike the Prairie soil the addition of inoculum to the Sandhill soil resulted in fungus mats and coralloid mycorrhizae with attached rhizomorphs in three of the 10 pots. These were chiefly ectotrophic Type 1 mycorrhizae although some Type 2 mycorrhizae were present as they were in many of the single and multiple forked roots in the other pots without coralloid mycorrhizae. Ectendotrophic mycorrhizae were found in only one section. The Sandhill soil to which fumigated inoculum was added (2B) remained free of mycorrhizae. The untreated inoculum (3A) produced some fungus mats, rhizomorphs and coralloid mycorrhizae of the ectotrophic Type 1 whereas none were found in the fumigated inoculum (3B).

The lack of any beneficial effect on growth by inoculation may have been due to the inhibition of the mycorrhizal fungi by the ex-

tremely high temperatures during the months of July and August. The high temperatures obviously did not eliminate the fungi as evidenced by the development of mycorrhizae in some of the pots. The rate of growth of the fungi, however, was obviously reduced as evidenced by the complete lack of any fungus mats or coralloid mycorrhizae when examined after 9 month's growth. There was eventual formation of some mycorrhizae in the inoculum and in the inoculated Sandhill soil during the last 3 months of the test with temperatures of 70° to 80° F., even though in many cases not developed to the point of forming coralloids. This raises the question of whether a more vigorous growth of the fungi under more favorable conditions earlier in the experiment would not have resulted in beneficial effects as in the previous experiment.

Fumigation with methyl bromide was found to be very effective in controlling mycorrhizal fungi. Such an effective fumigant should have promise for experimental work in which suspected mycorrhizal fungi are to be tested without introducing the additional chemical and physical changes occasioned by other means of disinfection or sterilization.

Judging by the results of this experiment, however, more investigations are needed on the possible effects of fumigation. In addition to the control of mycorrhizal fungi there was also control in Soil No. 1 of damping-off and root rot. It must also be remembered that other than the high temperature effect, fumigation was the chief difference between this experiment and the previous test. It is probable that the effects of fumigation on the subsequent soil microflora will vary in different soils and that in some soils such as the rich Prairie soil (No. 1) the ultimate effect may be to depress the growth of seedlings. At least that is one possible explanation of the divergent results of these two experiments with the same soils.

One effect of fumigation was evident soon after treatment in the growth of fungi on the surface of the soils. The fumigated duff-soil mixture had a heavy growth of *Trichoderma* and when added to the fumigated Prairie soil resulted in abundant growth of both *Trichoderma and Pyronema* whereas this did not occur with the Sandhill soil. Neither of the fumigated soils to which untreated inoculum was added showed such growth of fungi.

Effect of Mycorrhizae on Chlorosis

In a previous test it was noted that a large number of the seedlings growing in the Chestnut soil from western Nebraska developed much more chlorosis than the seedlings in the same soil to which 5 percent of inoculum had been added. In order to determine whether this was due to biological action, or to chemicals or nutritional action resulting from the addition of inoculum, another test was conducted using both fumigated and non-fumigated duff-soil inoculum. Twenty

seven-inch pots, each containing three seedlings, were used in each soil treatment and the fumigation was with methyl bromide. The seedlings were grown in a greenhouse maintained at 70° to 80° F. for 10 months.

At the end of two months chlorosis was starting to appear in the newly developing secondary needles. Notes taken at two-month intervals showed an increasing amount of chlorosis both as to the number of affected seedlings and the degree of severity in the untreated soil and in the soil supplemented with fumigated inoculum. Only a few seedlings appeared chlorotic in the soil to which untreated inoculum had been added and unlike those in the other soils the number did not increase and the severity of the few affected gradually decreased with the passage of time. Several of those showing chlorosis after four or five months appeared healthy at the end of the experiment.

Examination after five months failed to reveal any fungus mats, coralloids, or rhizomorphs on any of the root-balls in the untreated soil (Table 6, No. 1) or with the addition of fumigated inoculum (No. 3) whereas 10 of the 20 pots in the series to which untreated inoculum had been added (No. 2) showed good fungus mats and numerous coralloid mycorrhizae. At the end of the experiment only two of the 20 in this inoculated soil (No. 2) had failed to form mycorrhizae and it was interesting to note that the three seedlings in one of these pots were chlorotic and those in the other pot were small and unthrifty. The final notes taken after 10 months continuous growth are summarized in Table 6.

It is clearly evident from the data that inoculation with a duff-soil mixture resulted in fewer chlorotic seedlings and an increased vigor as measured by height, collar diameter and green weight. The differences in measurements would be even greater if the seedlings which had died of chlorosis had been included. The only chlorosis occurring in the inoculated soil was very mild. The results with the fumigated inoculum tend to eliminate the possibility of the ameliorating factor being other than biological. There was a constant associa-

Table 6. Relation of mycorrhizae to chlorosis of ponderosa pine seedlings.

Series No.	Soil Treatment[1]	Seedlings	Chlorotic	Surviving seedlings[2]		
				Ht.	Diam. collar	Green wt. tops
		No.	Pct.	mm.	mm.	gm.
1	Untreated	58	47	64	2.1	4.3
2	Inoculated	51	10	93	3.0	11.6
3	5% fumigated inoculum added	57	55	68	2.1	5.0

[1] The soil used in this test was the Chestnut soil as listed in Table 1.
[2] Four of the seedlings in Series 1 and one in Series 3 died of chlorisis and were not included in the measurements.

tion between the development of mycorrhizae, decreased severity of chlorosis and increased vigor of the seedlings.

Dale, McComb and Loomis (1955) report jack pine produced a normal growth on a high lime soil (pH 8.2) only when the soil was treated with humus from a healthy pine plantation with the resultant formation of mycorrhizae. Chlorosis occurred and mycorrhizae were absent in the same soil treated with various combinations of NPK fertilizers with and without micronutrients or with soil acidification or the addition of iron sulfate or chelated iron. It has often been considered that chlorosis on such high lime soils is due to a lack of available iron in the plant and can be corrected by the addition of iron or chelating agents. In their experiments, however, iron failed to control chlorosis and at the higher rates even appeared to be toxic.

The soil used in the present study, while having a relatively high pH (6.8) for pines, was from the upper 4 or 5 inches of a fine sandy loam in virgin grass and had no lime concretions present. At the end of the experiment these soils tested pH 7.2 to 7.4. No tests were conducted with iron or chelating agents and the exact nature of the physiological disturbance resulting in the chlorosis is not known. Examination of the roots of the chlorotic seedlings failed to reveal the presence of pseudomycorrhizae or other pathogenic fungi. The roots were healthy but were reduced in size and without mycorrhizae. It was evident, however, that normal healthy seedlings could be grown in this soil if mycorrhizal development occurs.

Relation of Mycorrhizae to Drought Tolerance of Pines

Tolerance to conditions of low rainfall is a critical factor in determining the tree species to be planted in shelter belts and farm windbreaks throughout the Great Plains. In Nebraska it had been found that over a 50 year period the survival of ponderosa pine in the Sandhill soil of the Nebraska National Forest at Halsey was better than that of other pine species tested. It is significant that *P. ponderosa* is native in Nebraska.

The ability of *P. ponderosa* to remove water from the soil even after the permanent wilting point of sunflower had been reached was shown by Fowells and Kirk (1945) with 1-year-old potted seedlings. Using two-year-old seedlings in sandy loam soil, Stone and Fowells (1955) found that the seedlings survived for 20 days after the death of sunflower and that this could be increased to 41 days in the presence of an artificial dew applied to the tops at night. Stone (1957) has also reported a difference in the survival time in a sandy as compared with a clay loam soil. The seedlings reduced the soil moisture content one percent below the permanent wilting point of sunflower in the sandy loam whereas in clay loam the seedlings died when the moisture content was still one percent above the permanent wilting point. Using three-year-old "re-potted" seedlings in Sheridan clay loam he

found *P. ponderosa* seedlings survived 64 days after the ultimate wilting point of sunflower had been reached and that the survival time was increased to 94 days in the presence of artificial dew at night. He considered from the above studies "that the seedlings of *P. ponderosa* have certain genetically fixed physiological characteristics that permit them to exist in soil after it has reached the permanent wilting point of sunflower, provided there is abundant nightly dew fall." He does not mention the presence of mycorrhizae in his experiments but the fact that he was using two-year-old seedlings supplied by the U. S. Forest Service and that the seedlings were well established before watering was discontinued would indicate that the roots were probably mycorrhizal.

The significance of mycorrhizae in relation to drought tolerance of pine was reported on by Cromer (1935) in Australia. Working with *P. radiata* and an ectendotrophic mycorrhiza he attributed to *Boletus granulatus,* he observed the effect of drought on mycorrhizal and non-mycorrhizal roots. He described a complete collapse of the cells of the cortex of non-mycorrhizal roots under drought conditions and their inability to recover when immersed in water. He failed to detect any corresponding shrinkage or collapse in the cortex of the mycorrhizae and stated that dessication of these rootlets did not take place and that a renewal of growth occurred within three days of an effective rainfall following a drought period when the soil moisture had fallen to a critical point for a month. He considered that the fungus was responsible for this protective action either due to the presence of the mantle or the ability of the fungus hyphae to absorb moisture at a lower moisture content than the normal root.

Cromer's observations were on the roots of mature 30-year-old trees and refer to the death or survival of individual roots of those trees. If this phenomenon also occurs in seedlings it could determine the death or survival of the seedling and would thus be a highly significant factor in the survival of seedlings being set out in the low rainfall areas of the Great Plains.

In an attempt to determine this possible effect of mycorrhizae on the drought tolerance of seedlings a preliminary test was made with 16-month-old seedlings growing in individual four-inch clay pots. Water was withheld for five weeks during a period of high daily maximum temperatures of 90° to 100° F. None of the seedlings, either with or without mycorrhizae, were able to recover from this severe test.

Another test consisted of 80 ponderosa seedlings in six-inch pots, two seedlings per pot, in inoculated Sandhill soil. One half of these were fumigated with methyl bromide before planting. After seven month's growth, water was withheld for 40 days from one half of each of these lots and the other half was kept at optimum moisture. Sunflowers which had been planted in the pots from which water was to be withheld were 6 to 15 inches high at the time watering stopped. After

two weeks the terminal leaves of these sunflowers reached a point of permanent wilt and soil samples from these dry soils showed only 1.28 percent moisture. The seedlings were kept at a temperature of 70° to 80° F. but again the test was too severe and none of the seedlings recovered when watering was renewed.

Many ectotrophic mycorrhizae were present on the roots in the unfumigated soils and none occurred in the fumigated. In the dry fumigated soils there was a complete collapse of the cortical tissue of the short roots as described by Cromer (1935). The mycorrhizal short roots in the dry, unfumigated soil also showed some collapse of the outer cortical cells but not as severe as in the non-mycorrhizal roots in the fumigated soils. The seedlings were unable to recover, however, from the drought injury.

Thirty-two of the remaining healthy plants in the moist soils were then divided into two sets and water was withheld from one set for a period of 20 days at which time the soil moisture had been reduced to 0.8 percent dry weight. The drought symptoms were the same as in the previous test and again there was no recovery when watering was renewed. In the moist unfumigated soil there were well developed fungus mats and coralloid mycorrhizae whereas in the dry set the coralloids had all turned black and there was no apparent renewal of growth of the root or of the fungus mantle which was present before watering was stopped. These old dead mycorrhizae were found to contain many dark collapsed cortical cells although there was a well developed fungus mantle and an extensive intercellular net.

One more test was made using somewhat similar methods but withholding water for a slightly shorter period of 17 days when the seedlings were 8 months old. These were in a Sandhill soil to which either fumigated or non-fumigated duff-soil inoculum had been added. Three seedlings were grown in each 7-inch clay pot at a temperature of 70° to 80° F. At the end of the 17-day period without watering the soil moisture averaged 0.87 percent.

The first drought symptoms were similar in both the mycorrhizal and non-mycorrhizal seedlings with the needles becoming greyish-green and drooping in contrast with the upright, bright green needles of the seedlings at optimum moisture. At the time watering was renewed there was very little browning of the needles and that was confined to the primary needles on a few of the non-mycorrhizal seedlings. During the following month all seedlings were kept at optimum soil moisture and the effect of the previous water shortage became increasingly evident.

Final notes were taken 45 days after watering was renewed. In the soil to which untreated inoculum had been added the root-balls from 13 of the 20 pots showed fungus mats and coralloid mycorrhizae whereas none were present in the soil to which fumigated inoculum had been added. In both soils all 54 of the seedlings growing in moist

soil were healthy with those in the mycorrhizal soil being slightly taller. Both sets of seedlings had increased about 11 percent in height during the 45 days.

In the pots from which water had been withheld for 17 days, only four of the 30 seedlings in the mycorrhizal soil failed to recover and three of these were in pots that failed to develop coralloid mycorrhizae. Twelve of the 29 seedlings in the non-mycorrhizae soil were dead. There was also a difference in the recovery of the surviving seedlings. There was more severe necrosis of the old needles on the seedlings in the non-mycorrhizal soil and less vigorous development of the terminal bud and new needles. The increase in height of the surviving seedlings during the last 45 days was 12 percent for those in the mycorrhizal soil compared to 8 percent for the non-mycorrhizal.

As in the previous experiment a microscopic examination of the short roots showed that there was a severe but not a total collapse of the cortical cells in the non-mycorrhizal seedlings. There was also a slight collapse involving only the outer cortical cells of the mycorrhizal roots. In the surviving seedlings it was found that a considerable number of the old brown to black forked roots of the mycorrhizal seedlings had renewed growth from the tips usually without any macroscopically visible fungus mantle. Only a very few of the old short roots in the non-mycorrhizal seedlings showed evidence of renewed growth.

Under the conditions of this experiment, with the sudden and drastic decrease in soil moisture, this difference in the ability of the short roots to renew growth did not appear of sufficient magnitude to be the sole factor in determining survival. The differences in the ability to form new roots were much greater and probably of more significance. In the mycorrhizal seedlings there were many new forked short roots developing on the laterals at the bases of the old, black, apparently functionless mycorrhizae. This type of development, which was rarely found in the non-mycorrhizal seedlings, was similar to that often seen in the field as previously described.

From the standpoint of total new root development, the greatest difference occurred in the production of new laterals which in turn produced new short roots. In the mycorrhizal seedlings many of the old secondary roots developed an abundance of new laterals. In the non-mycorrhizal seedlings there were relatively few new long roots and these usually arose from the tap root with none developing from the old laterals or secondary roots.

It thus appeared that survival depended more upon the ability of the long roots to withstand these severe drought conditions than upon the renewed growth of the short roots as emphasized by Cromer (1935). In this connection it is of interest to note that Robertson (1954) reports that in long roots in which the Hartig net is not present the cortical cells quickly lose their contents and collapse earlier and more extensively than when the cortex is infected. This could possibly be

a factor in the higher rate of survival of the long roots of the mycorrhizal seedlings in this drought test but the present evidence is insufficient to draw such a conclusion. It may well be that the greater ability to survive is determined by the sum total of all the mycorrhizal influences not the least of which would be larger, more vigorously growing seedlings with a better developed root system and presumably a greater quantity of reserves essential for new growth.

The results of these seedling tests are of course not directly comparable with Cromer's (1935) observations on mature trees. Neither can the high mortality in the relatively short period of moisture deficiency be compared with the results of Stone (1955). His tests dealt essentially with the ability of the seedlings to withdraw water from different types of soil in closed containers. The tests in the present study were designed primarily to determine the ability of the seedlings to withstand a sudden and severe water shortage caused not only by their own utilization of available soil moisture but also by the evaporation of moisture from the surface of the soil and through the porous sides of the clay pots. The soil used was also 92 percent sand with a moisture content of only 2.6 percent at 15 atmospheres.

The last test conducted was the only one that indicated a relative tolerance to soil moisture deficiencies by the mycorrhizal seedlings as compared with the non-mycorrhizal. While the results must be viewed with caution, because of the very severe conditions imposed on the seedlings and the small number of seedlings used, they would appear to justify further studies of this factor which can be of such great importance in the survival of transplanted stock in the Great Plains area. Large scale field experiments with mycorrhizal and non-mycorrhizal transplants in different soils with and without supplemental watering are needed.

DISCUSSION

The absence of mycorrhizal fungi from prairie and other grassland soils has been noted by workers in different parts of the world. This conclusion, based in many instances on observation and empirical tests, has been questioned by others who point to the lack of adequate experimental proof. Very few investigations have been reported from the vast grassland area of the Great Plains where many millions of nursery grown trees are planted each year and their survival is of great importance.

Rosendahl and Wilde (1942) and McComb (1943) have given evidence of the lack of mycorrhizae in the prairie soils of Wisconsin and Iowa. The present study confirms those results for widely different grassland soils in Nebraska. In only one instance were mycorrhizae found on pine seedlings growing in virgin grassland soil and that may have been due either to contamination in the open greenhouse flats used in a preliminary experiment or to the fact that the grassland may

have become infested with wind blown inoculum from a pine stand only 250 feet distant. Further experiments would be needed to confirm this later hypothesis which if true would raise the question of how long such fungi would survive in the absence of their higher symbiont.

No evidence was found to substantiate the theory that grassland soils may harbor soil microorganisms producing toxins which inhibit mycorrhizal fungi (Neilson-Jones, 1943). Neither was there evidence that with an adequate mineral nutrient supply it makes little difference whether or not roots are mycorrhizal (Davis, Wright and Hartley, 1942). In all but one of the present tests the addition of 5 percent duff-soil inoculum increased the vigor of the seedlings even on the rich prairie loam.

Practically all trees planted in grassland areas are nursery grown and therefore, except for the establishment of new nursery sites, whether or not tree mycorrhizal fungi are indigenous is of less importance than their ability to survive in their new environment. This raises many questions of the reputed high site requirements for specific mycorrhizal fungi as influenced by all the various biological and physical factors involved in the soil environment. The present study was of too short a duration to answer questions of survival on an experimental basis. The specific fungi involved were not determined and therefore their reaction to the various environmental factors is unknown. It was obvious, however, that several different fungi were present and that the two predominant ones were the basidiomycetous type of fine mycelium associated with the ectotrophic mycorrhizae and the coarse mycelial type always found with the ectendotrophic forms.

The ideal procedure in studying the influence of different soils and other factors on the type of mycorrhizae produced would be to have a standard of comparison based on the forms of mycorrhizae found in natural stands of the same species. Obviously, it is impossible to set up such a type collection in a treeless area and the only recourse is to examine the mycorrhizae on the roots of nursery transplants which have survived for many years. The types of mycorrhizae described in this study were found in common association in all of the collections from nursery sites to stands 25 to 50 years old on a wide variety of soils. They also occurred on seedlings grown in the greenhouse on different types of inoculated grassland soils. They were also present on a number of different species of pine. The origin of some of the older stands in the Nebraska National Forest is somewhat obscure, but it is known that some were pulled native seedlings from the Black Hills of South Dakota and others were nursery grown seedlings from Minnesota and other nurseries in the Great Plains area. It is possible that the present forms may have been the dominant ones at the time of introduction, or it may be that they were the only ones capable of surviving in the new environment, or perhaps they are variants of the

originals which through selective action have become the dominant ones under the new conditions.

The common occurrence of ectendotrophic mycorrhizae is at variance with the experience of workers in the forested areas of eastern United States (Hacskaylo 1957, Hacskaylo and Palmer (1957-a) but is in agreement with McComb's (1943) findings in Iowa. Doak's (1934) incomplete description of the type of infection caused by a fungus resembling *Rhizoctonia sylvestris* on several pine species resembles the ectendotrophic form described in the present study. He also reported it as infecting the cortex of mother roots. The theory that the ectendotrophic forms constitute a transition stage between ectotrophic and endotrophic mycorrhizae has been advanced by some workers, but there was no evidence found in these tests to support this assumption.

It was not uncommon to find in one collection or on the roots of the same tree, the typical ectendotrophic form and others which were indistinguishable, on the basis of both gross and microscopical characters, except for the absence of the intracellular mycelium. This fact and the more common occurrence of the intracellular mycelium in the older cortical tissue suggests that we may be dealing with an ectotrophic fungus which under certain conditions becomes the dominant partner in this delicately adjusted host-parasite relationship and penetrates intracellularly. Such a question can only be resolved by pure culture isolations, so far unsuccessful, and the synthesis of fungus and host under carefully controlled experimental conditions. It should not be forgotten, however, that such studies do not prove that the fungus in question is the predominant one in nature or even that it would be capable of acting in the same manner in competition with other fungi in different soils and under variable environmental conditions. There is great need for a type of study that is conspicuously absent from the literature which would follow the synthetic production of mycorrhizae with transplants of such seedlings into different soils under a variety of conditions with a careful investigation of the sequence of development of new mycorrhizae under these more exacting competitive requirements.

The recurring evidence in the present study of renewed growth of mycorrhizae is at variance with the widely accepted view that mycorrhizae are ephemeral or annual structures. This regeneration occurred not only in the spring as a renewed growth of the mycorrhizae formed the previous year, but also as successive elongations of either simple or forked short roots in greenhouse grown seedlings. It may be that different fungi are involved than those so extensively studied in other areas where this type of development apparently is not a common occurrence.

Explanations of this renewed growth being due to changes in the metabolism of the tree, and also of the fungus at different seasons of the year and under different conditions would at first glance be an in-

adequate explanation of such development under fairly constant conditions in the greenhouse. Basically, however, the same factors might well be involved. Even under such conditions top growth of seedlings does not follow a logarithmic growth curve but occurs as successive surges of growth. This apparent rhythmic periodicity of shoot development is probably paralleled by the root development and would indicate profound changes in the rate of metabolic activities which would in turn affect the delicate balance between root and fungus. The observations here reported indicate a need for a great deal more emphasis being placed on this phase of the development of mycorrhizae.

No evidence could be found in this investigation to support the statement made by Davis, Wright and Hartley (1942) that "short roots that are not mycorrhizal are predominantly pseudomycorrhizal," and that "When pine seedbeds are placed on soil that has not recently grown pines, these pseudomycorrhizae may be the dominant form during the first 2 or more years." The seedlings grown in these tests on virgin grassland soils failed to produce pseudomycorrhizae, or at least none were detected, and none were found in any of the field collections. There were many forked short roots on healthy seedlings that fitted the published descriptions, but microscopic examination failed to reveal any that appeared to be pathogenic. The occurrence of pseudomycorrhizae appears to be well established in Great Britain and while there are numerous reports of their occurrence in the United States they are based primarily on gross morphological characters unsupported by the isolations and inoculations needed to prove their identity and pathogenicity. The present study emphasizes the unreliability of gross characteristics particularly when unsupported by any evidence of harmful effects. Under the conditions of these experiments and field observations there was nothing to indicate that pseudomycorrhizae are a problem either for the establishment of nurseries on grassland soils or in the survival of transplants used for the establishment of shelter belts and windbreaks.

The ability of many mycorrhizal fungi to live saprophytically in the soil has been questioned by many workers, and their dependence on an association with their tree hosts has often been stressed. At the same time it is commonly asserted that the long roots are not internally infected and that the mycorrhizal short roots are ephemeral structures. The observation that the fungus spreads from short root to short root by hyphal wefts or rhizomorphic hyphal strands is hardly an adequate explanation of the multitude of mycorrhizal short roots found on pine in nature, sometimes under conditions where hyphal wefts and rhizomorphs are not present. Robertson's (1954) evidence of the longitudinal extension of the fungus through the cortex of the long root and the infection of the emerging short root raises many questions regarding the interpretation of previous work in which the

influence of various factors was evaluated in terms of the number of mycorrhizal short roots. The possibility of air borne inoculum as evidenced by other data he presents opens up still other questions regarding conclusions reached in previous experimentation.

The work herein reported, while not designed to study these two points, does confirm Robertson's evidence of the internal infection of laterals and extends it to include the ectendotrophic mycorrhizae. The possibility of air borne inoculum cannot be excluded as one explanation of the only instance of extensive mycorrhizal development in a virgin grassland soil that occurred in these tests (p. 27).

The renewed growth of persistent mycorrhizae for more than one year, which was of common occurrence with the brown, smooth ectendotrophic form, presents still another type of mycorrhizal development. All three of these possible methods of perpetuation of mycorrhizal fungi added to the previous common concepts of infection help to provide a more adequate explanation of the ecological status of mycorrhizae. It is clear that much more work remains to be done in those phases of mycotrophy which have been somewhat neglected in recent years for the more exact methods of physiological experimentation and the use of synthetic cultures. More information is needed regarding the occurrence, sequence of development and structural characteristics of mycorrhizae as influenced by various physical and biological variants in order to properly apply the results of the physiological investigations.

It has been shown that in certain non-mycorrhizal soils that tend to produce chlorotic seedlings the condition can be corrected and healthy green seedlings can be grown if mycorrhizal fungi are introduced. The identity of the fungi that produce this change is not known, nor do these limited experiments show whether or not they would survive indefinitely in these soils. Dale, McComb and Loomis (1945) state that in Iowa, nursery stock with mycorrhizal roots planted on high-lime soils may become chlorotic and die within a 3- to 5-year period or with chlorosis developing more slowly may persist for 10 to 15 years. In western Nebraska chlorosis has not been a serious problem either in native stands or with transplanted nursery stock in windbreaks even though many of the soils are quite alkaline. It may be that the mycorrhizal fungi that exist in the nurseries with alkaline soils are more capable of surviving than are the mycorrhizal fungi existing in the acid soils of nurseries further east. The need for more exhaustive studies on the ecological status of the mycorrhizal fungi is again clearly indicated.

SUMMARY

1. The occurrence of mycorrhizae on pines, chiefly *P. pondersoa,* in Nebraska was found to be relatively sparse as compared with descriptions of their occurrence in the forested areas of eastern United States.

2. They were produced more abundantly in the fall than in the spring at which time their development was rather late in relation to top growth.

3. In many soils they were found only at considerable depth, particularly in the spring, and in compacted soils distichous mycorrhizae were formed.

4. Rather than being ephemeral annual structures, many mycorrhizae persist for at least two years with successive elongations. Renewed growth from the tips of old mycorrhizae is also evident in the greenhouse under uniform conditions for continued growth for the period of the experiments (up to 18 months).

5. Ectendotrophic mycorrhizae without macroscopically visible mantles were of common occurrence.

6. Ectotrophic mycorrhizae associated with a basidiomycetous fungus was the most prevalent form with coralloids, mantles and rhizomorphs. Another ectotrophic form associated with an apparently different, but undetermined fungus, occurred usually without coralloids or macroscopically visible mantles.

7. Both inter- and intracellular mycelium were found in long roots and were continuous with the mycelium in the developing mycorrhizal short roots.

8. No structures which could be considered pseudomycorrhizae were encountered in this study.

9. Mycorrhizae became established in virgin non-mycorrhizal grassland soils by inoculation with a duff-soil mixture. These were Prairie, Sandhill and Chestnut soils with pH's ranging from 5.8 to 6.8 and of widely different physical and chemical structures. Mycorrhizae were present in nursery soils ranging up to pH 7.8.

10. The addition of duff-soil inoculum to non-mycorrhizal virgin grassland soils under conditions which permitted the development of the mycorrhizal fungi usually resulted in a variable increase in the growth of seedlings on different soils, but the beneficial effect if any, was not in inverse ratio to the fertility of the soil.

11. Severe chlorosis of seedlings in some virgin grassland soils was prevented or lessened in severity by inoculation which resulted in the formation of mycorrhizae.

12. More mycorrhizal than non-mycorrhizal seedlings were able to survive a rapid and extreme soil moisture deficiency due to withholding all watering for 17 days. There was no survival in either group of seedlings when water was withheld for periods of 20 to 40 days.

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